CHAPTER 5. DETECTION, ISOLATION AND IDENTIFICATION OF *ESCHERICHIA COLI* O157:H7 AND O157:NM (NONMOTILE) FROM MEAT PRODUCTS (Revision # 1; 9-6-99)

William C. Cray, Jr., Douglas O. Abbott, Frankie J. Beacorn, and Steven T. Benson

5.1 Introduction

The following method is used for the analysis of raw and ready-to-eat meat products for *Escherichia coli* O157:H7 and O157:NM (O157:H7/NM). The method is based on enrichment in a selective broth medium, application of a rapid screening test, immunomagnetic separation (IMS) in paramagnetic columns, and plating on a highly selective medium.

This method represents a modification of the previous FSIS methodology. As such, this method supercedes all previous FSIS *E. coli* 0157:H7/NM laboratory methods including MLG Chapter 5; DETECTION, ISOLATION AND IDENTIFICATION OF *ESCHERICHIA COLI* O157:H7 AND O157:NM (NONMOTILE) FROM MEAT AND POULTRY PRODUCTS by Amelia K. Sharar and Bonnie E. Rose.

5.2 Safety

E. coli 0157:H7/NM is a human pathogen with a low infectious dose. (Ingestion of 100 cells can cause disease.) The use of gloves and eye protection is mandatory, and all work surfaces must be disinfected prior to and immediately after use.

5.3 Materials Required

5.31 Equipment

a. Balance, sensitivity of 0.1 g
b. Stomacher™ 400 or 3500 with appropriate sizes of sterile Stomacher™ bags, with or without mesh. (Tekmar Co., Cincinnati, Ohio), or equivalent
c. Incubator, static 35 $\pm$ 2°C
d. Micropipettors to deliver 15-1000 µl with sterile disposable filtered micropipet tips
e. Mechanical Pipettor with 1.0 ml, 5.0 ml, 10.0 ml sterile pipets
f. Inoculation loops, “hockey sticks” or spreaders, and needles

g. UV light (long-wave)
h. Filter unit, 0.2 µm, nylon, sterile
i. Infrared thermometer
j. LabQuake® Agitator (or equivalent) with clips to hold microcentrifuge tubes
k. Sterile disposable 12 x 75 mm polypropylene tubes (e.g. Fisher # 14-956-1B, or equivalent)
l. Sterile capped tubes to hold at least 5 ml
m. Microcentrifuge and sterile 1.5 ml microcentrifuge tubes
n. Sterile 50 ml conical tubes (e.g. Falcon # 2070, or equivalent)
o. Sterile 40 µm Cell Strainer (Falcon # 2340, or equivalent)
p. MACS Large Cell Separation Columns (Miltenyi Biotec # 422-02, or equivalent)
q. OctoMACS Separation Magnet (Miltenyi Biotec # 421-09, or equivalent)
r. Multistand to support OctoMACS Separation Magnet (Miltenyi Biotec # 423-03, or equivalent)
s. Tray, autoclavable, approximately 130 mm x 83 mm (e.g. VWR # 62663-222, or equivalent)for use with the OctoMACS

5.32 Media / Reagents / Cultures

a. Modified EC broth with novobiocin (mEC+n) (or equivalent)
b. Rainbow® Agar 0157 (Biolog Inc., Hayward California, 94545) containing 10 mg/L novobiocin plus 0.8 mg/L potassium tellurite, or equivalent selective medium
c. Ewing's motility test medium (0.4% agar)
d. Modified Ewing's motility test medium for motility enhancement (0.35% agar)
e. Sheep Blood Agar plates
f. SOB + A Medium
g. E Buffer, approximately 7 ml per sample [Buffered Peptone Water, Bovine Albumin Sigma # 7906 (or equivalent) , and Tween-20, or equivalent]
h. Lysol, 2.0%, or equivalent
i. Dynal # 710.04 anti-*E. coli* O157 antibody-coated paramagnetic beads (Dynal Inc., Lake Success, NY 11042), or equivalent
j. *E. coli* O157:H7 strain 465-97 (positive control)
k. *E. coli* ATCC strain 25922 (negative control)
l. Triple sugar iron (TSI) agar
m. Cellobiose fermentation broth with Andrade's indicator (Ewing, 1986)

5.33 Test Kits

a. ELISA-based screening test for the detection of *E. coli* O157:H7/NM. This test should meet or exceed the following performance characteristics:

Sensitivity	≥98%
Specificity	≥90%
False Negative Rate	≤ 2%
False Positive Rate	≤10%

b. *E. coli* O157:H7 latex agglutination test kit (RIM® *E. coli* O157:H7 Latex Test Kit, REMEL, 12076 Santa Fe Drive, Lenexa, KS 66215, or equivalent)

c. Biochemical test kits and systems [Vitek GNI and GNI Plus cards (bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395) or equivalent]

5.4 Detection Procedure

a. Sample Preparation

Note: The enrichment broths should be allowed to warm to at least 18◦C prior to use.

i. Raw ground beef microbiological testing programs. Randomly collect five 65-g sub-samples (total of 325 g) from as many sites as possible in the package of raw ground beef. Place each 65-g sub-sample in a sterile Strainer Stomacher™ bag. Add 585 ml mEC+n broth and pummel for two minutes in a Stomacher™.

ii. Semi-dry and dry fermented sausages. Aseptically remove the exposed cut surfaces and cut the sausages into small cubes consisting of both shell and core. Weigh a sufficient number of the cubes into five sterile Stomacher™ bags to make five 65-g sub-samples. Prepare a 1:10 dilution by adding 585 ml of mEC+n broth to each sub-sample. Pummel for 2 minutes in a Stomacher™.

iii. Outbreak-related samples and cooked meat patties. Randomly collect thirteen 25-g sub-samples (total of 325 g) from as many sites as possible in the package(s) of meat. Place each 25-g sub-sample in a sterile Strainer Stomacher™ bag and add 225 ml of mEC+n broth. Pummel for 2 minutes in a Stomacher™.

b. Incubate all bags (static) with their contents for 20 to

24 h at 35 ± 2°C. Include a positive, negative, and uninoculated medium control for each group of samples tested at one time. Use a fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97) as a positive control and *E. coli* ATCC strain 25922 as the negative control.

c. From the enrichment cultures in the Stomacher™ bags, perform the ELISA-based *E. coli* O157:H7/NM screening test following the manufacturer's directions. To prevent clogging the pipette tip, be sure to collect the appropriate size sample from the enrichment broth outside the inner strainer bag.

d. Samples negative by the screening test can be reported as negative for *E. coli* O157:H7/NM and discarded.

e. Samples positive by the screening test should be reported as potential positives. Isolation and confirmation can be initiated from the enrichment culture in the Stomacher™ bag.

5.5 Isolation Procedure

a. Prepare E buffer by mixing 0.5 g Bovine Albumin and 50 µl Tween-20 into 100 ml Buffered Peptone Water (BPW). Filter sterilize (0.2 µm) and store at 2-8°C.

b. Remove Dynal #710.04 Beads from 2-8°C storage and place on ice.

c. Remove Rainbow® Agar plates from 2-8°C storage, allowing 3 plates for each screen-positive culture and each control. Plates should be dried for up to 30 minutes in the laminar flow hood (with the lids removed) prior to use. Dried plates that are not used should be labeled "dried", placed in bags and returned to 2-8°C.

d. Remove a bottle of E Buffer from the refrigerator. Decant approximately 7 ml of E Buffer for each culture and each control into a separate 50 ml tube and allow it to warm to at least 18°C. (Return the stock E Buffer to 2-8°C.)

e. Place the positive control, negative control and enrichment broth cultures to be assayed in a tube rack. Order and label the tubes so that the positive control is first, followed by the negative control, then all

cultures. Maintain this order for subsequent steps.

f. For each positive control, negative control, and culture, label two sterile 1.5 ml microcentrifuge tubes, one 50 ml conical centrifuge tube and two 12 x 75 mm capped tubes. For each pair of 12 x 75 mm tubes, label one tube "1:10" and add 0.9 ml E buffer (for step s).

g. Prepare the Dynal #710.04 *E. coli* O157:H7 immunomagnetic bead suspension in a microcentrifuge tube according to Table #1 below. Be sure to include the positive and negative controls in the total number of cultures. Return the stock vial of Dynal #710.04 *E. coli* O157:H7 immunomagnetic beads to 2-8°C.

h. Vortex the bead solution briefly (2-3 seconds), then add 50 µl to labeled microcentrifuge tubes (one per culture and controls). Hold these tubes on ice.

i. Collect approximately 5 ml from each enriched culture and control into separate sterile capped tubes for use in the IMS.

j. For each positive control, negative control, and culture, place a 40 µm Cell Strainer on a 50-ml conical centrifuge tube. Vortex each tube briefly, pour the contents through the respective filter and collect at least 1.0 ml of filtrate.

k. The OctoMACS magnet will hold up to 8 tubes, therefore proceed with 8 or fewer culture tubes. Transfer 1.0 ml of a filtrate (step j) to the corresponding microcentrifuge tube (step h) and place in the clips of the LabQuake® tube agitator. Rotate the tubes for 10 - 15 min. at 21-24◦C.

l. Attach the OctoMACS magnet to the Multistand.

m. Position a tray on the base of the Multistand so that it will collect the filtrate passing through the columns. Add approximately 300 ml of 2% Lysol to cover the bottom of the tray.

n. Carefully open as many as 8 Large Cell Separation Column packages. Label and place the columns on the OctoMACS magnetic collector. Insert columns from the front making sure the column tips do not touch any surfaces. Leave the plungers in the bags at this time to maintain

sterility.

o. Pipet approximately 500 µl E buffer to the top of each column and let the buffer run through.

p. Vortex, then pipette each culture and control from step k, to its corresponding column.

q. After a culture or control has drained through, wash the column by applying 1.0 ml of E buffer to each column and allow to drain. Repeat 3 more times for a total of 4 washes.

r. After the last wash has drained, remove the column from the OctoMACS magnet and insert the tip into an empty labeled 12 x 75 mm tube. Apply 1 ml of E buffer to the column, and using the plunger supplied with the column, *immediately* flush out the beads into the tube. Use a smooth, steady motion to avoid splattering. Cap the tubes. Repeat this for each column. If there are more than 8 cultures and controls, decontaminate the OctoMACS (as in step w) after each set of 8 has been processed. Repeat steps k-r for the additional cultures.

s. Vortex the tubes from step r briefly to resuspend the beads. Make a 1:10 dilution of each treated bead suspension by adding 0.1 ml of the bead suspension to a 12 x 75 mm labeled tube containing 0.9 ml E Buffer.

t. Vortex briefly to maintain beads in suspension and plate 0.1 ml from each tube (steps r and s) onto a labeled Rainbow® Agar plate. Use a hockey stick or spreader to spread plate the beads, being careful not to spread the beads against the edge of the plate.

u. Vortex the tubes containing undiluted beads from step r and centrifuge one minute using a bench-top microcentrifuge to concentrate the beads. Withdraw and discard the supernatant without disturbing the beads. Add 0.1 ml of E buffer to the beads, vortex to resuspend, and pipette the suspension to a labeled Rainbow® Agar plate. Spread plate the beads as described in step t.

v. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 24 - 26 h at 35 $\pm$ 2◦C.

w. Decontaminate the OctoMACS magnetic collector by applying 2% Lysol directly to the surface. After approximately ten minutes, rinse with deionized or tap water. Allow the unit to air-dry or use absorbent paper towels to dry the unit.

5.6 Identification and Confirmation

a. After incubation, *E. coli* O157:H7 colonies have black or gray coloration on Rainbow® Agar. When *E. coli* O157:H7 colonies are surrounded by pink or magenta colonies, they may have a bluish hue. Mark colonies typical of *E. coli* O157:H7 and perform latex agglutination assays for 0157, following manufacturer's instructions. Streak all latex positive colonies, up to a total of five from each sample (one per sub-sample, if possible) onto Blood Agar plates. Incubate Blood Agar Plates for 16 - 20 h at 35 $\pm$ 2°C.

Note: Hold the original Rainbow® plates at 20-24°C until results are confirmed. If no latex-positive colonies were found at 24 h, re-examine the plates for typical colonies after an additional 6 – 20 h.

b. After incubation, examine the Blood Agar plates for purity under visible light and evidence of cross contamination with the positive control under UV light. If the Blood Agar Plates appear pure and uncontaminated, perform the following confirmatory tests:

i. Biochemical confirmation.
Inoculate Vitek-GNI or GNI Plus cards. In order to differentiate between *E. coli* 0157 and similar competitive organisms, include the following conventional tubed media: TSI, cellobiose, and motility test medium.

ii. Serological confirmation.
To confirm the absence or presence of 0157 and H7 antigens, use an *E. coli* O157:H7 latex test agglutination kit (RIM® *E. coli* O157:H7 Latex Test Kit, or equivalent). Use growth from the blood agar plate and follow the manufacturer's directions.

c. If the isolate confirms as an *E. coli* 0157:H7, it may be reported positive at this point. If the culture is non-motile, the H7 test is negative or nonspecific, or

(optionally) the H7 test is positive, perform tests for the presence of Shiga-like toxin(s) and/or the toxin gene(s).

If the Shiga-like toxin(s) and/or one or more toxin genes are present, the sample will be treated as positive for *E. coli* 0157:H7 or *E. coli* 0157:NM and regulatory action will be taken. The cultures will also be tested by pulsed-field gel electrophoresis (PFGE) for potential epidemiological association.

5.7 Quality Control

In addition to the requirements listed in MLG, Ch.36, it should be noted:

a. Rainbow® Agar plates have a shelf life of 2 weeks.

b. All media must be pre-warmed to 18–35°C prior to use.

c. The recommended fluorescent strain of *E. coli* O157:H7 must be used in this procedure to monitor for cross contamination. The protocol for the use of fluorescent strains of *E.coli* O157:H7 as positive controls follows:

Wild-type strains of *E. coli* O157:H7 transformed with pGFP produce a green fluorescent protein. As a result of this transformation, fluorescent strains of *E. coli* O157:H7 possess the unique property of expressing bright green fluorescence visible in the dark when illuminated by long-wave UV light. This property, which sets them apart from typical *E. coli* O157:H7, makes them useful positive controls for analyses of meat samples for *E. coli* O157:H7/NM. At different steps in the procedure, both test samples and (fluorescent) positive controls can be tested for the bright green fluorescence as a Quality Control measure to make sure that positive sample isolates actually came from the test sample and not from accidental contamination by the positive control cultures.

Results of studies done at the FSIS Beltsville Microbial Pathogens Laboratory showed that these fluorescent cultures can be subjected to *E. coli* O157:H7/NM isolation and identification procedures without losing their fluorescent properties. These strains retain their fluorescent properties when grown in SOB media with added ampicillin (SOB + A). These cultures must be transferred

every 5 days to fresh SOB + A media, according to the protocol outlined below. The fluorescent colonies are ready to be used as positive controls on day 3 of the following protocol, and for the next 4 consecutive days without losing their fluorescent properties. If these cultures are not needed on a continuous basis, they can be stored at refrigeration temperatures on SOB + A agar plates in zip-lock bags or sealed with parafilm for 1 month and then transferred, or started up again 2 days before needed. ***Strict adherence*** to the protocol described below is essential, in order to ensure that the fluorescent strains do not lose their ability to express green fluorescence.

i. Test the fluorescent *E. coli* O157:H7 strain (FSIS culture # EC 465-97 or the currently designated control strain) on SOB + A agar plate for fluorescence by illuminating colonies under long-wave UV light in the dark.

ii. Select only fluorescing colonies and inoculate into 10 ml of SOB + A broth in a tube. Incubate at 35 $\pm$ 2°C overnight.

iii. Streak the culture from the SOB + A broth onto SOB + A agar plate. Incubate at 35 $\pm$ 2°C overnight.

iv. Examine colonies on the plate for fluorescence. The fluorescent colonies are ready to be inoculated into modified EC broth + novobiocin (mEC+n) at this stage. These cultures on SOB + A agar plates can be stored refrigerated and be used as positive controls for 4 more days. Incubate the inoculated mEC+n positive control culture at 35 $\pm$ 2°C overnight, along with the test samples.

v. Continue analysis per Sections 5.4 - 5.6 above and test the Blood Agar Plates of the fluorescent positive controls and any positive sample cultures for fluorescence.

Table 1 Calculations for immunocapture beads						
# of Cultures	*ul Beads **	*ul E-Buffer*		*# of Cultures*	*ul Beads **	*Ul E-Buffer*
1	15	135		26	145	1305
2	20	180		27	150	1350
3	25	225		28	155	1395
4	30	270		29	160	1440
5	35	315		30	165	1485
6	40	360		31	175	1575
7	45	405		32	180	1620
8	50	450		33	185	1665
9	55	495		34	190	1710
10	60	540		35	195	1755
11	65	585		36	200	1800
12	70	630		37	205	1845
13	75	675		38	210	1890
14	80	720		39	215	1935
15	85	765		40	220	1980
16	90	810		41	230	2070
17	95	855		42	235	2115
18	100	900		43	240	2160
19	105	945		44	245	2205
20	110	990		45	250	2250
21	120	1080		46	255	2295
22	125	1125		47	260	2340
23	130	1170		48	265	2385
24	135	1215		49	270	2430
25	140	1260		50	275	2475

*** Dynal anti-*E. coli* 0157:H7 antibody-coated paramagnetic beads (vortex briefly before use)**

5.8 Selected References

Ewing, W. H. 1986. Edwards and Ewing's Identification of Enterobacteriaceae, 4th Edition. Elsevier Science Publishing Co., Inc., New York.

Fratamico, P. M., M. Y. Deng, T. P. Strobaugh, and S. A. Palumbo. 1997. Construction and characterization of *Escherichia coli* O157:H7 strains expressing firefly luciferase and green fluorescent protein and their use in survival studies. J. Food Prot. 60:1167-1173.

Harrison, B. and D. Warburton. 1997. Identification of *Escherichia coli* verotoxins by the Meridian Premier EHEC kit®. Laboratory procedure MFLP-93 in *The Compendium of Analytical Methods*, Vol. 3. Health Protection Branch, Health Canada, Ottawa, Canada.

Hitchins, A. D., P. Feng, W. D. Watkins, S. R. Rippey, and L. A. Chandler. 1995. *Escherichia coli* and the coliform bacteria. Chapter 4 in FDA bacteriological analytical manual, 8th ed., p. 4.23. AOAC International, Gaithersburg, MD.

Hitchins, A. D., P. A. Hartman, and E. C. D. Todd. 1992. Coliforms-*Escherichia coli* and its toxins, p. 325-369. *In* C. Vanderzant and D. F. Splittstoesser (ed.), Compendium of Methods for the Microbiological Examination of Foods. 3rd Edition. Amer. Publ. Hlth. Assoc., Washington, D.C. 20005.

Okrend, A. J. G., B. E. Rose, and B. Bennett. 1990a. A research note: A screening method for the isolation of *Escherichia coli* O157:H7 from ground beef. J. Food Prot. 53:249-252.

Park, C. H., K. M. Gates, N. M. Vandl, and D. L. Hixon. 1996. Isolation of Shiga-like toxin producing *Escherichia coli* (O157 and non-O157) in a community hospital. Diagn. Microbiol. Infect. Dis. 26:69-72.

Taormina, P. J., M. Rocelle, S. Clavero, and L. R. Beuchat. 1998. Comparison of selective agar media and enrichment broths for recovering heat-stressed *Escherichia coli* O157:H7 from ground beef. Food Microbiol. 15:631-638.

Weagant, S. D., J. L. Bryant, and K. G. Jinneman. 1995. An improved rapid technique for isolation of *Escherichia coli* from foods. J. Food Prot. 58:7-12.